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Effect of transient focal ischemia on blood-brain barrier permeability in the rat: correlation to cell injury

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Abstract Prolonged ischemia is known to damage the blood-brain barrier, causing an increase in vascular permeability to proteins. We studied the time course of extravasation of endogenous albumin in rats after 1 and 2 h of middle cerebral artery (MCA) occlusion followed by 6, 12, and 24 h of recirculation. In a separate group of rats that had undergone 1 h of MCA occlusion and 6 h of recirculation, influx of [^{14}C]aminoisobutyric acid (AIB) from blood to brain was also measured. After 1 h of occlusion followed by 6 h of recirculation, neuronal damage was evident in caudoputamen, but there were no signs of blood-brain barrier leakage to either AIB or albumin. At 12 h, the caudoputamen contained extravasated albumin, and at 24 h extravasation was extended to the somatosensory cortex. Animals subjected to 2 h of MCA occlusion showed albumin extravasation in caudoputamen already at 6 h of recirculation, and at 12 and 24 h albumin was abundant in the major part of the right hemisphere. This study suggests that damage to neurons precedes leakage of the blood-brain barrier. Even a relatively short period of ischemia such as 1 h will result in markedly increased vascular permeability. However, a longer transient ischemic insult disrupts the blood-brain barrier earlier than a shorter one.

Key words Brain · Focal ischemia · Reperfusion · Albumin extravasation · Blood-brain barrier

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Introduction

Several groups have investigated the integrity of the blood-brain barrier during focal ischemia [4, 7, 14, 20, 23, 26]. In cases of stroke early edema is of the cytotoxic type, but permanent focal ischemia of longer than 6 h duration will also damage the blood-brain barrier [16, 26] and give rise to an increase in vascular permeability to large molecules, such as proteins. When the ischemia is transient, vascular dysfunction appears earlier, and has been shown to be more severe [7, 20, 26]. An open question is whether endothelial cell damage, and thereby blood-brain barrier dysfunction, precedes neuronal injury after transient ischemia, or if primary ichemic cell damage is followed by loss of vascular integrity.

In this study, we examined the integrity of the blood-brain barrier after transient focal ischemia of short duration (1–2 h), and explored whether there was a correlation between the development of tissue damage and blood-brain barrier dysfunction. For this, we studied the time course for extravasation of endogenous albumin after 1 and 2 h of middle cerebral artery (MCA) occlusion followed by 6, 12, and 24 h of recirculation, and evaluated morphological tissue damage in the ischemic focus (lateral caudoputamen and somatosensory cortex), and the penumbral area (motor cortex).

Materials and methods

Animals and operative techniques

Fasted male Wistar rats (Møllegaard's Breeding Center, Copenhagen, Denmark) were used for this study. The NIH principles of laboratory care were followed, and the experiments were approved by the Ethical Committee for Laboratory Animal Experiments at the University of Lund. Focal ischemia was induced by occlusion of the right middle cerebral artery with an intraluminal filament [15, 17–19] under halothane anesthesia. The animals were allowed to recover from anesthesia, and adequate occlusion of the MCA was confirmed by the occurrence of a neurological deficit, with the animals circling to the left [2]. After 1 or 2 h blood flow was restored to the ischemic area by withdrawal of the occluding fila-

ment under renewed anesthesia. The animals were thereafter allowed recirculation periods of 6, 12, or 24 h.

Immunohistochemistry

At the end of the recirculation period (6, 12, and 24 h, $n = 8$ in each group), the animals were again anesthetized, and the brains were perfusion-fixed with formaldehyde, sectioned coronally in 2.8-mm-thick blocks, dehydrated, and embedded in paraffin. Coronal brain sections of 5-µm thickness taken at the level of bregma, the region which usually shows the largest infarct area in this model, were used for immunohistochemical visualization of extravasated serum albumin using the avidin-biotin-peroxidase complex (ABC) method [13]. In brief, the sections were placed on chrome-gelatine-covered glass slides, deparaffinized, and rehydrated. Thereafter, the sections were rinsed in TRIS-HCl buffer solution (TBS), and incubated in 0.4% pepsin in TBS for 15 min at 37°C to unmask antigen sites, followed by rinsing in TBS. To eliminate endogenous peroxidase activity, the sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol. To reduce the nonspecific protein binding, the sections were incubated for 2 h at room temperature with 1% chicken egg albumin in TBS. The sections were then incubated for 48 h at 4°C with the primary antibody, rat albumin antiserum, diluted to 1:8000 in 1% chicken egg albumin, followed by incubation with the biotinylated secondary antibody and ABC (Vectastain, ABC-kit, Vector Laboratories, Burlingame, Calif.). The bound antigen-antibody-ABC complex was visualized using 3,3'-diaminobenzidine as a chromogen. The sections were counterstained with hematoxylin-eosin, and evaluated by light microscopy.

Aminoisobutyric acid penetration

Four animals subjected to 1 h MCA occlusion followed by 6-h recirculation were re-anesthetized, and the integrity of the blood-brain barrier to aminoisobutyric acid (AIB) was tested as follows. A bolus of 15 µCi [^{14}C]AIB diluted in 1.0 ml saline was injected i.v., and was allowed to circulate for 20 min. Arterial plasma samples (20 µl) were repeatedly collected to allow integration of specific activity. At the end of the experiment, the animals were decapitated, and their brains were removed and frozen in 2-methylbutane chilled to –50°C. Tissue samples, weighing 10–15 mg, were dissected at –15°C from the ischemic focus (lateral caudoputamen) and penumbra (motor cortex) of the MCA-occluded hemisphere (Fig. 1) to allow assessment of tissue tracer activity.

The plasma-to-brain AIB transfer coefficient, K (µl · g/min) was calculated as described by Blasberg et al. [5] using the following equation:

$$K = \frac{C^*_{br}(T)}{\int_0^T C^*_{pl}(t)\,dt}$$

in which C^*_{br} is the brain tissue concentration of radioactivity after 20 min, and C^*_{pl} is the plasma activity. The cerebral blood volume was set to 0.01 ml/g tissue, according to Ohta et al. [21], to allow correction for AIB remaining in the vasculature.

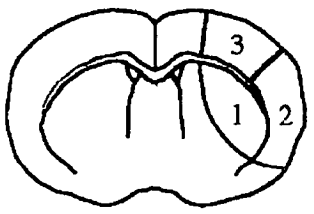

Fig. 1 Schematic drawing of the section used for histological evaluation of albumin extravasation. *1* Lateral caudoputamen (ischemic focus), *2* cortical focus, *3* cortical penumbra. Tissue samples for measurement of aminoisobutyric acid influx were taken from areas *1* and *3*

Results

Physiological parameters

Blood pressure, body temperature, blood gases and pH were measured just before and 10 min after the MCA occlusion, and were found to be within the normal ranges in all animals.

Albumin leakage

Three patterns of albumin incorporation into tissue were seen, all of which were usually present in the same animal: (1) vague neuropil staining, evenly distributed in the damaged area; (2) normally shaped neurons taking up albumin in the cytoplasm; and (3) shrunken necrotic neurons containing albumin in the whole cell.

Albumin leakage was mainly confined to the ischemic area, with the earliest changes seen in the ischemic focus, while prolongation of recirculation caused the changes to spread into penumbral tissues. After 24 h of recirculation the area of albumin extravasation and the area containing necrotic neurons/infarction were closely overlapping. In the 1-h MCA occlusion group, neuronal damage preceded albumin leakage in the ischemic focus.

Caudoputamen, 1-h MCA occlusion

No sign of blood-brain barrier leakage to albumin was found after 6 h of recirculation, but neuronal damage was evident to a mild to moderate extent (Fig. 2A). After 12 h of recirculation, the caudoputamen contained albumin, mainly distributed around vessels, and taken up by dying neurons (Fig. 2B). After 24 h of recirculation all neurons, including the still normal-shaped, large caudoputaminal neurons, contained albumin (Fig. 2C).

Cortex, 1-h MCA occlusion

After 6 h of recovery the neocortex had a completely normal appearence; after 12 h two animals were still unaf-

Fig. 2A–D Photomicrographs from caudoputamen, stained for visualization of endogenous albumin in tissue. **A** After 1-h MCA occlusion plus 6 h of recirculation, many shrunken, damaged neurons (some marked by *arrowheads*), but no albumin staining, are seen. **B** After 1-h MCA occlusion plus 12 h of recirculation, albumin is visible as brown staining in the neuropil around the vessel (*V*), and has been taken up by damaged neurons (some marked with *arrows*). **C** After 1-h MCA occlusion plus 24 h of recirculation, all neurons in the caudoputaminal focus have incorporated albumin and show still normal-shaped large neurons, known to be less sensitive to ischemia (*arrow*). **D** After 2-h MCA occlusion plus 6 h of recirculation, i.e., after a longer insult, albumin uptake is already seen in neurons showing ischemic cell changes (some marked with *arrows*). A–D Hematoxylin-eosin plus the avidin-biotin-peroxidase (ABC) method for albumin staining. *Bar* = 50 µm

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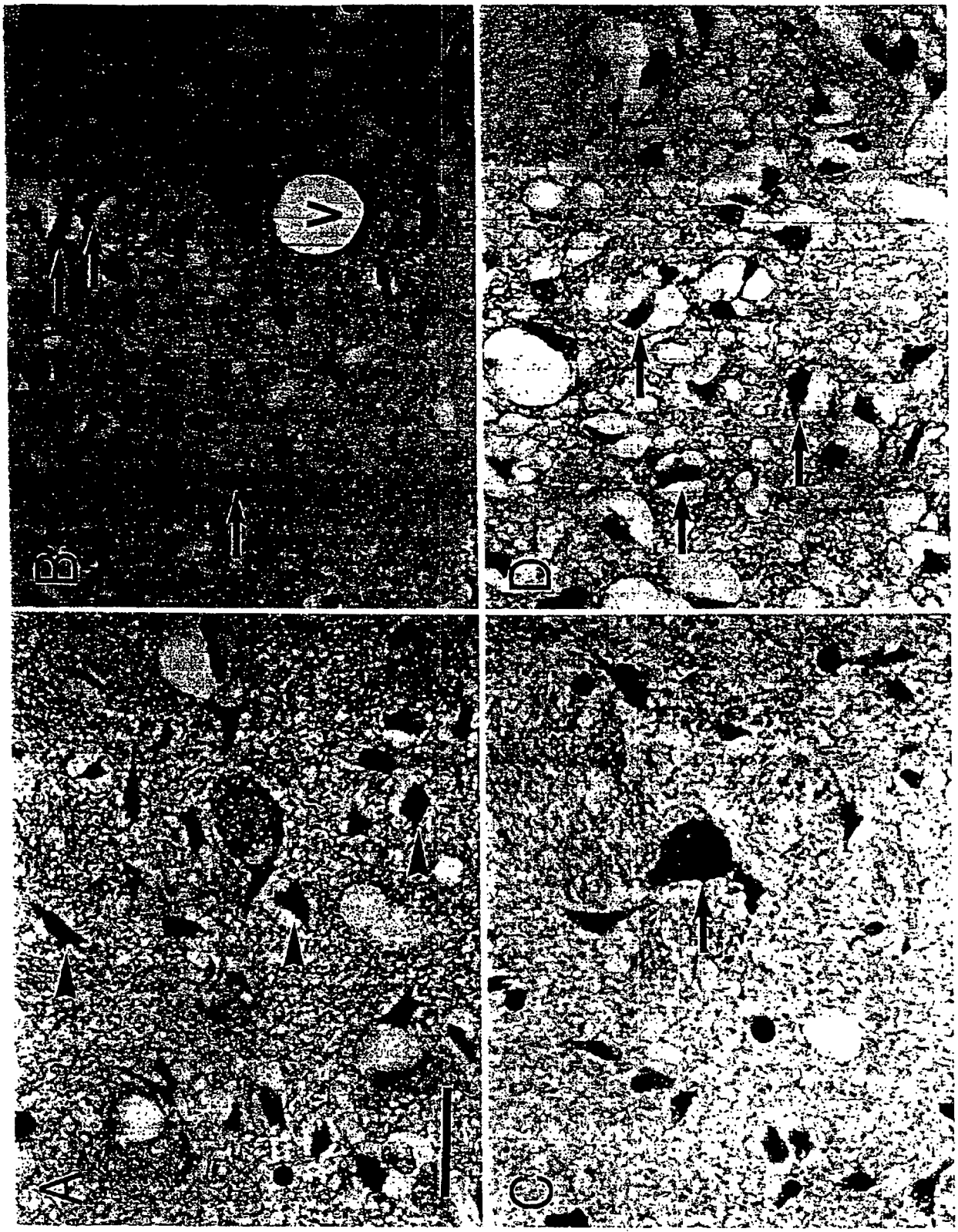

Fig. 3A–C Photomicrographs of the cortical penumbra at different times of recirculation after 2 h of MCA occlusion. A No injured neurons and no extravasation of albumin is seen at 6 h of recirculation. B At 12 h of recirculation the neuropil show a diffuse staining for albumin, and some neurons exhibit ischemic cell changes without albumin incorporation (*arrowheads*). C After 24 h of recirculation all tissue components in the severely damaged tissue contain albumin. A–C Hematoxylin-eosin plus the ABC method for albumin staining. *Bar* = 50 μm

fected and the remaining six showed pale albumin staining and necrotic neurons in the somatosensory cortex (cortical focus). The pattern after 24 h of recirculation was similar to that at 12 h, with two animals without detectable damage or albumin extravasation, and the remaining exhibiting diffuse albumin staining and damaged neurons often containing albumin.

Caudoputamen, 2-h MCA occlusion

Albumin extravasation was seen already at the earliest recirculation time studied, 6 h. Neuronal damage in the lateral caudoputamen was dense, and the shrunken neurons contained albumin (Fig. 2D). After 12 and 24 h, serum albumin was abundant in both neuronal and glial compartments in this structure (not shown).

Cortex, 2-h MCA occlusion

The somatosensory cortex, directly overlying the caudoputaminal ischemic focus, constitutes the cortical focus which during ischemia suffers a blood flow reduction similar to that of caudoputamen. Changes in the somatosensory cortex followed a pattern similar to that seen in the caudoputamen after 2-h ischemia, with early albumin extravasation and the presence of necrotic neurons.

Fig. 4 Cortex at 24 h of recirculation following 2 h of MCA occlusion: the border between normal tissue (*N*) and infarct (*INF*) is sharply demarcated both in terms of tissue damage and of albumin content. Note, however, that several damaged neurons in the borderline do not contain albumin (*arrowheads*). Hematoxylin-eosin plus the ABC method for albumin staining. *Bar* = 100 μm

In the cortical area with less dense ischemia during the MCA occlusion (the penumbral zone, see Fig. 1), no injured neurons and no extravasation of albumin were seen at 6 h after 2-h transient focal ischemia (Fig. 3A). After 12 h of recirculation some neurons showed ischemic cell changes, mainly without incorporation of albumin, and the neuropil had started to show a diffuse staining for albumin. After 24 h of recirculation the whole tissue was severely damaged and all tissue components contained albumin with the exception of a few glial cells (Fig. 3C). At this time, the border of the infarct was very demarcated both in terms of tissue damage and of albumin content (Fig. 4).

[^{14}C]Aminoisobutyric acid transfer

The transfer coefficient (K) for AIB from blood to brain after 6 h of recirculation following 1 h of MCA occlusion was 0.82 ± 0.54 and 1.23 ± 0.86 µl · g/min in the ischemic focus and penumbra, respectively. These values were not statistically different from those measured in normal control animals in corresponding areas (0.92 ± 0.14 and 1.07 ± 0.34 µl · g/min).

Discussion

In this study, despite the manifest neuronal damage, the blood-brain barrier was still intact to serum albumin, as well as to smaller molecules such as AIB, at 6 h after a transient focal ischemia of 1-h duration. With longer recirculation times (12 h) leakage to albumin became evident in the brain areas showing tissue damage.

When the duration of ischemia was prolonged to 2 h, albumin extravasation was seen already after 6 h of recirculation in tissues subjected to dense ischemia, i.e., the caudoputamen and somatosensory cortex, and exhibiting pronounced neuronal necrosis, while in the cortical penumbral area albumin was not extravasated until neuronal damage started to occur.

These findings lead us to conclude that it is unlikely that a dysfunction of the blood-brain barrier, to an extent which allows large molecules to penetrate, is involved in the development of neuronal necrosis.

Several studies have shown that the blood-brain barrier is intact to proteins, and also to smaller molecules such as AIB, for 4–6 h after permanent focal ischemia [3, 4, 16, 22–24]. Ischemic edema is formed before this time, however, due to an increased influx of sodium and chloride into the tissue. When reperfusion is instituted after 2–3 h of focal ischemia, vascular dysfunction is aggravated, and vasogenic edema ensues, adding to an already increased water content of the tissue [7, 16, 20, 26].

Our results demonstrate that the blood-brain barrier permeability to albumin increases at a time when neuronal damage is already manifest. A morphological study by Garcia et al. [11] supports this finding, in showing that neuronal necrosis becomes prominent 6 h after permanent MCA occlusion in rats, i.e., at a time point at which the blood-brain barrier is intact [4, 26]. Furthermore, Tamura et al. [25] studying focal ischemia in cats, demonstrated that only animals with severe tissue damage showed blood-brain barrier disruption, while subjects with mild or no damage had an intact barrier to Evans blue. However, the extent of damage in that study was very severe, including hemorrhagic infarcts in three out of four cats with Evans blue leakage, rendering the information less conclusive. Thus, to our knowledge, the present atudy is the first to demonstrate that neuronal damage precedes, and even may be a prerequisite for a blood-brain barrier opening following transient focal ischemia.

The mechanisms behind the breakdown of the barrier following ischemia remains unclear, but an improvement of barrier stability has been shown in situations which are associated with nerve cell protection, such as treatment with a nitric oxide synthase inhibitor [20], as well as with hypothermia [14], while aggravation of blood-brain barrier dysfunction has been seen as an effect of platelet-activating factor [6] and arachidonic acid metabolites [1, 7]. Taken together, these studies suggest that the cascade of events following massive glutamate release during ischemia is ultimately involved in creating endothelial cell damage. The cellular origin of deletarious molecules formed by such mechanisms, and exerting their action on the vasculature, is not known. They may be formed in neural tissue or in vascular cells. In the latter case, aggregation of platelets, and expression of adhesion molecules causing leukocyte sticking to the vessel wall ([9], for reviews see [8, 10, 12]), may cause microcirculatory disturbances and further increase endothelial injury. However, more research is needed to determine whether the endothelial damage originates from injury to neural tissue or to the vasculature.

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